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Periodate oxidation of marine high molecular weight dissolved organic matter: Evidence for a major contribution from 6-deoxy- and methyl sugars

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Abstract

Nuclear magnetic resonance spectroscopy has been used to infer that marine high molecular weight dissolved organic matter (HMWDOM) is a mixture of carbohydrates, proteins, and lipids. However, acid and base catalyzed hydrolysis of HMWDOM followed by molecular level analyses using gas and liquid chromatography provide only low to modest yields of simple sugars, amino acids, and lipids. Here we use periodate over-oxidation to investigate the composition of HMWDOM. Our analyses show that the oxidation of HMWDOM carbohydrate consumes more periodate per carbon on a molar basis (1.3:1) than simple sugars (0.8:1) or linear polysaccharides (1.0:1), and that HMWDOM is highly branched. We also recover acetic acid and methanol as major oxidation products. Methanol and acetic acid are derived from the oxidation of methyl and 6-deoxy sugars, and our analyses suggest these sugars are major components of HMWDOM. Periodate over-oxidation shows that lipids are not major constituents of HMWDOM, and that most of the alkyl carbon observed in the ^{13}CNMR spectra between 0–45 ppm that has been previously assigned to lipids is due to the methyl carbons of acetamide and 6-deoxy sugars.

Key words: Dissolved organic carbon; Carbohydrates; Sugars; Lipids

1. Introduction

Nearly all (>99%) organic carbon in the ocean is sequestered as dissolved organic matter (DOM). The residence time and broad outline of DOM cycling is known through natural abundance radiocarbon measurements (Druffel et al., 1992), but the composition, sources, and fates of DOM remain poorly understood. Approximately 30% of DOM can be sampled by ultrafiltration, which retains organic matter >1 nm, and thereby concentrates the high molecular weight DOM (HMWDOM) fraction. Nuclear magnetic resonance (^{13}C NMR, ^{1}HNMR) spectra of HMWDOM in surface seawater show at least 60–70% of HMWDOC is carbohydrate (Benner et al., 1992; Aluwihare et al., 1997, 2002). Treatment of HMWDOM with strong acid should depolymerize HMWDOM carbohydrate to simple sugars, which can be quantitatively analyzed by a number of molecular level techniques including gas and high pressure liquid chromatography (GC, HPLC). However, the application of a broad suite of hydrolysis

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protocols yield only modest amounts (5–20% total HMWDOC) of largely neutral sugars from HMWDOM (McCarthy et al., 1996; Aluwihare et al., 1997; Borch and Kirchmann, 1997; Panagiotopoulos and Sempere, 2005). Most HMWDOM carbohydrate appears to be impervious to acid hydrolysis, and has yet to be characterized at the molecular level. The discrepancy between spectroscopic-based and hydrolysis/chromatography-based assessments of HMWDOM composition is a major impediment to understanding DOM composition and cycling. To more fully describe the composition of the HMWDOM carbohydrate fraction, strategies that do not rely on acid hydrolysis must be developed.

Other than carbohydrate, HMWDOM consists of acetate bound as acetamide to amino sugars as well as proteins, peptides, lipids and humic substances (Druffel et al., 1992; McCarthy et al., 1996; Aluwihare et al., 1997; Mannino and Harvey, 1999; Aluwihare et al., 2005). ^{1}HNMR spectra of HMWDOM isolated from surface seawater have a nearly constant ratio of resonances assigned to carbohydrate (3–5.5 ppm), acetate (2 ppm), and “lipid” (0.9–1.5 ppm), which led Aluwihare et al. (1997) to suggest that these three constituents are part of a common biopolymer that is a remnant of microbial production. Acetate can be quantitatively recovered as acetic acid from HMWDOM after acid or base hydrolysis (Aluwihare et al., 1997, 2005), but attempts to characterize the “lipid” fraction have not been as successful. Organic solvent extraction of saponified HMWDOM and subsequent analyses by GC–MS yield only small amounts of sterols and normal alcohols, fatty acids, and hydrocarbons (Mannino and Harvey, 1999; Aluwihare, 1999; Wakeham et al., 2003). Taken together, these lipids account for $<1\%$ of the HMWDOC, and most of these lipids may be only associated with HMWDOM rather than chemically bound into the HMW fraction (Boehm and Quinn, 1973). An alternative interpretation of the NMR spectral data is that the alkyl portion of the spectrum (1.3 ppm, ^{1}HNMR, 15–18 ppm ^{13}CNMR; Fig. 1) is due to contributions from 6-deoxy sugars. The methyl group in fucose and rhamnose, two common 6-deoxy sugars that have been previously identified in HMWDOM, appear at 15–18 ppm in ^{13}CNMR spectra and as 6 Hz coupled doublets at 1.3 ppm in the ^{1}HNMR spectrum (Bock and Thøgersen, 1982). If a significant fraction of the uncharacterized carbohydrate in HMWDOM is fucose or rhamnose, then these sugars could account for the unidentified “lipid” component.

In this study, we use periodate over-oxidation to better characterize the carbohydrate and lipid portion of HMWDOM. Periodate is a mild and selective oxidizing reagent that is widely used in carbohydrate structural analyses (House, 1972; Pazur, 1986). Periodate oxidation has been used to determine the degree of polymerization and branching in polysaccharides (Brown et al., 1948; Parrish and Whelan, 1959), the structure of aminosugars and lipopolysaccharides (Niemann and Hays, 1940; Perry and MacLean, 1999), and the presence or absence of glycoprotiens in DOM (Yamada and Tanoue, 2003). The selectivity of the oxidation provides controlled cleavage of C–C bonds between vicinal diols, yielding characteristic low-molecular weight products for simple neutral (formaldehyde [HCO], formic acid [HCOOH]), amino (ammonia), 6-deoxy (acetic acid) and methyl (methanol) sugars. Periodate oxidation can therefore reveal the presence of 6-deoxy and methyl sugars within the non-hydrolyzable HMWDOM carbohydrate fraction. For simple sugars such as glucose, periodate oxidation uses five moles of periodate to oxidize each mole of hexose (Sklarz, 1967). Conventional methods of periodate oxidation do not oxidize glycosidic bonds between sugars. However, periodate oxidation performed with an excess of periodate, at higher temperatures, and for longer reaction times does oxidize glycosidic linkages to formic acid or carbon dioxide. The moles of periodate consumed depends on the number of glycosidic linkages oxidized, but the stoichiometric ratio of periodate to hexose will be greater than five for an apparent “over-oxidation” of the polysaccharide (Whelan, 1964; Fig. 2). The stoichiometry of periodate over-oxidation can be

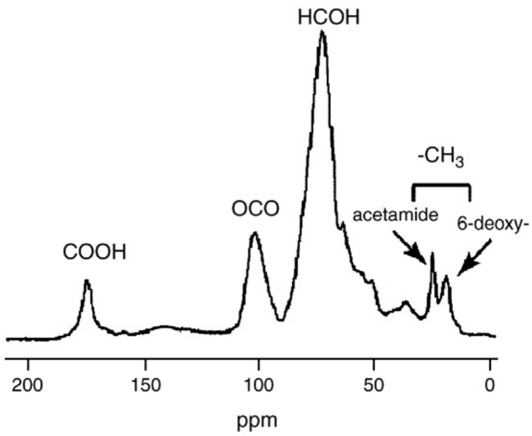

Fig. 1. ^{13}CNMR spectrum of HSUR02 showing the presence of carboxyl (COOH; 175 ppm), anomeric (O–C–O; 110 ppm), carbohydrate alcohol (HC–OH; 70 ppm) and lipid or alkyl carbon (CH_{2-3}; 15–25 ppm). Periodate oxidation suggests that the sharp “lipid” carbon resonance at 23 ppm is the methyl group of acetamide bound as *N*-acetyl amino sugars, and that the resonance at 17 ppm is due to 6-deoxy sugars.

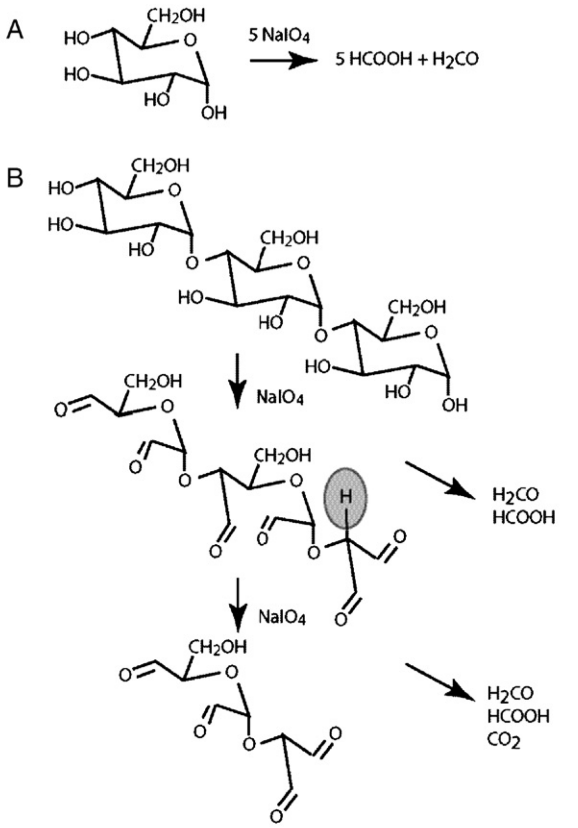

Fig. 2. (A) Periodate oxidation of glucose, and (B) periodate over-oxidation of maltotriose. The oxidation of oligo- and polysaccharides proceeds as in glucose first through the oxidation of vicinal alcohols to yield a polyaldehyde (middle structure). Oxidation of an acidic hydrogen (middle structure, circled) to an alcohol allows the oxidation to proceed to completion.

used to both to confirm that a large fraction of non-hydrolyzable HMWDOM is carbohydrate, and to characterize the degree of branching in this fraction (Brown et al., 1948). Finally, periodate does not oxidize saturated lipids (House, 1972). If a large fraction of HMWDOM is lipid, then periodate oxidation will release the lipid fraction and make the lipids available for GC- and HPLC-MS analyses.

2. Methods

Low carbon deionized (Milli-Q) water was used in all procedures. Glassware and sodium sulfate were combusted for a minimum of 8 h at 450 °C, and pipette tips, syringes and teflon-lined vial lids were rinsed with 10% HCl and water before use. Only amber colored or foil covered vials were used for all periodate reactions due to the sensitivity of periodate to light. Proton NMR spectra were acquired on a Bruker 400 MHz spectrometer using a water suppression program (zgpr). All ^{1}HNMR spectra were run using D_2O as a solvent, and chemical shifts were referenced to water at 4.8 ppm. Solid-state ^{13}CNMR spectra were acquired at 100.02 MHz. Samples were packed into a 7 mm zirconia rotor and spun at the magic angle at 7000 Hz. We used a cross polarization pulse sequence with a 1 ms contact time, and collected between 44,800–50,000 scans per sample. Spectra were processed with 10 Hz line broadening. Elemental analyses (CHN) were performed on a CE Instruments (Flash EA 1112) elemental analyzer with a thermal conductivity detector.

2.1. Sample collection

Seawater was drawn from the 15 m seawater intake at the Natural Energy Laboratory in Kona, Hawaii in January 2001 (HSUR01) and February 2002 (HSUR02). The samples were filtered to remove bacteria and small particles using a cleaned (10% HCl) Suporflow dual stage (0.8 μm and 0.2 μm) Gelman polyether sulfone cartridge filter (Chisolm Corp., Lincoln, RI) fitted to an Advanta stainless steel housing. Cell counts (DAPI stain) to quantify the contribution of bacteria to HMWDOM were not made on these samples, but counts made on similar samples show the filtrate contains $<10^7$ cells L^{-1}, and 2×10^9 cells L^{-1} in the 1000 fold concentrate. Assuming bacteria contain 12 fg C cell^{-1} (Fukuda et al., 1998), the typical contribution of bacterial carbon to the concentrate is approximately 24 μg C L^{-1}, or about 0.02% of the HMWDOC.

High molecular weight DOM samples were collected using a large volume cross flow ultrafiltration system consisting of a stainless steel centripetal pump and membrane housings and a fluorinated high density polyethylene reservoir. The system was plumbed with teflon tubing and fitted with PVDF valves. The ultrafiltration membrane (Separation Engineering, Escondido, CA) nominally retains organic matter with a molecular weight of greater than 1 kDa (>99% rejection of vitamin B_{12}). Membranes were cleaned using isopropanol, detergent (0.01% micro), HCl (0.01 N) and NaOH (0.01 N), stored in sodium azide (0.55 mM), and rinsed with water immediately before use. Samples were collected daily and frozen. Over the course of 10 days, a total volume of between 30,000 and 60,000 L of seawater was concentrated to approximately 20 L, frozen and returned to Woods Hole for further processing. The HMWDOM samples were desalted by diafiltration against 20 L Milli-Q water (added 10 times serially, with 2 L each time) until the addition of 2–3 drops of $AgNO_3$ (5 mg/50 mL water) to a 5–10 mL

Table 1
Elemental composition (%C) and monosaccharide distribution (mol%) in HMWDOM

	HSUR01	HSUR02	NPSG5200
%C	35	38	21
Rhanmose	11	11	15
Fucose	19	20	26
Arabinose	10	10	8
Xylose	13	11	9
Mannose	13	13	19
Galactose	18	18	12
Glucose	12	12	12
Glucosamine	3	4	ND [a]
Galactosamine	2	2	ND
Sugar (% HMWDOC)	16	12	3

[a] Not detected.

aliquot gave no visible precipitate. The desalted sample was then reduced to 2 L, and lyophilized to a fluffy white powder.

A deep water sample from 5200 m (NPSG5200), was taken from the North Central Pacific (31°00′ N, 159°00′ W) in June 1999. The sample was collected using rosette mounted Niskin bottles, filtered (0.2 μm Criticap polycarbonate filter cartridges), then processed using a spiral wound 1 kDa nominal molecular weight cutoff filter (Amicon Corp.) mounted on an Amicon DC-10 pump (Aluwihare et al., 2002).

2.2. Periodate oxidation

Commercially available sugars (glucose, laminarin [(1,3)-*O*-β-D-glucose], amylose starch [(1,4)-*O*-α-D-glucose], maltooligosaccharide [(1,4)-*O*-α-D-glucose]), protein (bovine serum albumin), and pentanoic (valeric) acid were used to test the periodate oxidation procedure and to confirm the stoichiometry of periodate oxidation. Calculations of molarity and molecular ratios for polysaccharides (laminarin, starch, and maltooligosaccharide) were based on the molecular weight of 162 Da (“dehydrated” glucose). Albumin concentrations were based on the average molecular weight of individual amino acids present (110 Da) instead of the total weight of the albumin protein (66 kDa). High molecular weight DOM samples were treated as glucose linear polysaccharides with molarity and stoichiometric ratios based on an assumed molecular weight of 162 Da.

Oxidations were run according to the procedure of Dixon and Lipkin (1954). Briefly, a 12–14 fold excess of 0.015 M aqueous solution of sodium (meta) periodate (Fisher Chemical Corp.) was combined with a 0.015 M aqueous solution of glucose, polysaccharide, bovine serum albumin, pentanoic acid or HMWDOM and oxidized in triplicate at 40 °C (glucose) or 80 °C (laminarin, maltooligosaccharide, amylose, albumin, pentanoic acid, HMWDOM) for 10–14 days. Only small amounts of sample NPSG5200 were available for this study, and this sample was oxidized with the same ratio of sugar to periodate, but as a more dilute solution (0.004 M glucose equivalents). The method for periodate measurement was adapted from the spectrophotometric analysis procedure of Hay et al. (1973). Changes in the concentration of periodate were determined by UV–Vis spectroscopy using a HP8452 diode array spectrophotometer. Absorbance spectra between 190 and 820 nm were measured daily, and the reaction was considered complete when the calculated percent oxidation was greater than 97%, or the change in absorbance values was less than 5% from the previous day. The fraction periodate consumed by the reaction at time t (F_t) was calculated as the ratio of blank corrected absorbance at 230 nm (B_t) to the initial periodate blank (P_0) where $F_t = B_t/P_0$ (Dixon and Lipkin, 1954). The maximum absorbance for periodate occurs at 223 nm, but a slight drift (+/−2 nm) in the periodate maximum during reaction was detected. In addition, the periodate reduction product (iodate) has a maximum at 190 nm. To avoid the influence from the shoulder of the iodate peak, determinations of the amount of glucose oxidized and the extent of reaction were calculated using the off-maximum absorbance values at 230 nm only. Variations of absorbance values for the periodate and glucose blanks were <5% throughout the course of the reaction. The stoichiometry of oxidation was calculated assuming complete oxidation of each substance.

Samples analyzed using both NMR and UV/Vis spectroscopy were prepared as described above, but with 99.9% D_2O as the solvent. Measurements were made daily for the first 3 days, then every second day

Table 2
The molar ratio of periodate:glucose for the oxidation of glucose, glucose linear polysaccharides, pentanoic acid and albumin

	Periodate: glucose	Periodate: carbon
Glucose	5	0.8
Amylose	6.7±0.4 (*n*=3) [a]	1.1
Maltooligosaccharide	6.2±0.5 (*n*=6)	1.0
Laminarin	5.8±0.9 (*n*=3)	0.97
Polysaccharide average	6.2±0.5 (*n*=12)	1.0
Pentanoic acid	0 (*n*=1)	0
Albumin	0.6±0.1 [b] (*n*=3)	0.1

For polysaccharides, glucose is calculated as moles of glucose equivalents assuming a molecular weight of 162 Da.

[a] Number of replicate analyses.

[b] Ratio of periodate to amino acid, assuming an average molecular weight of 110 Da for each amino acid and a C/N of 4.5.

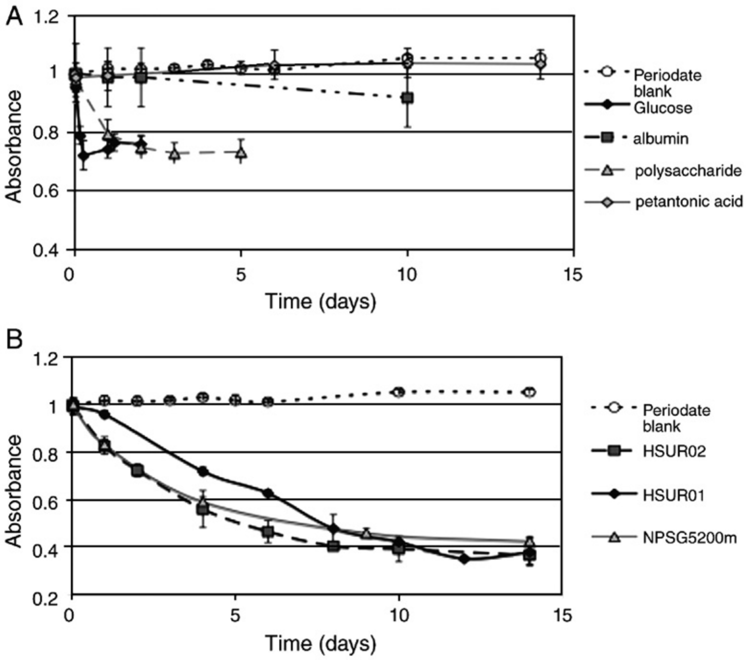

Fig. 3. Average normalized absorbance for (A) standards and (B) HMWDOM samples. Absorbance was measured via UV–Vis spectroscopy at 230 nm. Measurements were normalized to the 1 h periodate blank absorbance for each experiment, then averaged for substrate type. Error bars are standard deviations from the average. (A) Shows the absorbance averages for the periodate blank (open circles), glucose (filled diamonds), albumin (shaded squares), average polysaccharide (shaded triangles), and pentanoic acid (shaded diamonds). (B) Shows the absorbance averages for the periodate blank (open circles), HSUR02 (squares), HSUR01 (diamonds), and NPSG5200 m (triangles).

until the reaction was considered complete. Subsamples for NMR analyses were spiked with 10–50 μL of a 0.022 M benzoic acid as a reference standard. The amount of carbohydrate (3–5.5 ppm), acetate (2 ppm) and "lipid" (0.9–1.5 ppm) were determined by measuring the changes in ^{1}HNMR peak areas for each component relative to the benzoic acid standard over the course of the experiment.

2.3. Chemical analyses

Monosaccharides were analyzed by gas chromatography as their alditol acetates (Aluwihare et al., 2002). Briefly, 2 mg of HMWDOM (0.8 mg HMWDOC) were hydrolyzed with 0.5 mL of 2 M trifluoroacetic acid heated to 121 °C. After 2 h, the samples were dried and reduced to alditols by sodium borohydride. Alditols were acylated using acetic anhydride and 1-methyl imidazole and analyzed on a HP 5960 gas chromatograph equipped with a Supelco SP-2330 (30 m, 0.25 mm ID, 0.2 μm film) column and an FID detector. The temperature program was 55/20/150/4/240(15) (initial *T*/ramp/*T*/ramp/final *T* (hold time)), where all of the temperatures (*T*) are in degrees Celsius, ramps are in °C/min, and hold time is in minutes. The detector temperature was set at a constant 250 °C.

Lipids released during periodate oxidation of HSUR02 were analyzed by NMR spectroscopy and gas

Table 3
Periodate over-oxidation of HMWDOM

Sample	Mass (mg)	% Carbon	HMWDOC (μmol)	Periodate (μmol) consumed	Periodate: HMWDOC (μmol:μmol)
HSUR01	1.08	35	31.5	31.8	1
HSUR01	1.2	35	35	27.5	0.8
HSUR01	14.9	35	434.6	599.5	1.4
HSUR01	15.0	35	437.5	558.5	1.3
HSUR01	15.0	35	437.5	531.5	1.2
HSUR01	14.8	35	431.7	553.4	1.3
Average					1.2
HSUR02	54.8	38	1735	2216	1.3
HSUR02	55.3	38	1751	2236	1.3
HSUR02	14.9	38	471.8	609.7	1.3
HSUR02	14.9	38	471.8	600.4	1.3
HSUR02	15.0	38	475.0	608.0	1.3
HSUR02	14.6	38	462.3	611.4	1.3
Average					1.3
NPSG5200	7.3	21	127.8	172	1.3

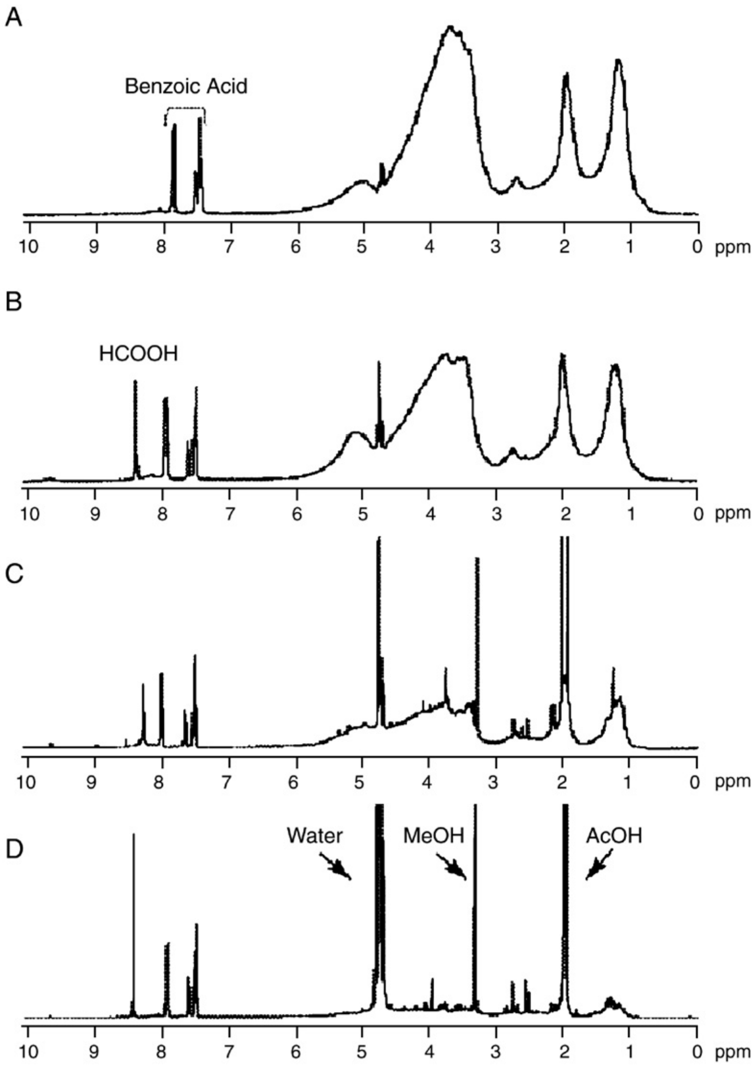

Fig. 4. ^{1}H NMR spectra for the periodate over-oxidation of HSUR02. The initial spectrum (A) was taken before the addition of periodate. The other spectra were taken after 1 h (B), 2 days (C) and 10 days (D) of oxidation. Benzoic acid was added to each sample immediately before acquisition of the NMR spectra as an internal reference standard. As oxidation proceeds, carbohydrate and aliphatics are degraded to formic acid, methanol, and acetic acid. The broad peak at 2 ppm in the sample before oxidation is due to acetamide bound as *N*-acetyl amino sugar. Periodate oxidation destroys the amino sugar releasing acetic acid which appears as a sharp singlet in the reaction product.

chromatography. After oxidation was complete, the pH of the sample was adjusted to pH=3 and the lipids extracted with 1.5 mL perdeuterated dicholoromethane. NMR spectra were taken of the aqueous and the organic phases. The organic extract was transferred into a GC vial, evaporated under nitrogen, and persilylated by reaction with *N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 15 min at 70 °C. The sample was then analyzed on a HP 6890 GC/5973 MS using a DB-XLB column (J and W Scientific, 60 m/0.25 mm ID/0.25 μm film). Injection was splitless and the temperature program was 50(1)/10/320 (37) (initial *T* (hold time)/ramp/final *T* (hold time)), where all of the temperatures (*T*) are in degrees Celsius, ramps are in °C/min, and hold time is in minutes.

3. Results

Monosaccharide analysis of the HMWDOM samples show approximately equimolar concentrations of seven neutral sugars: rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose (Table 1). The molar distributions of these seven sugars are similar to previous reports (Aluwihare et al., 1997). Neutral sugars were 12–16% of the total organic carbon in surface

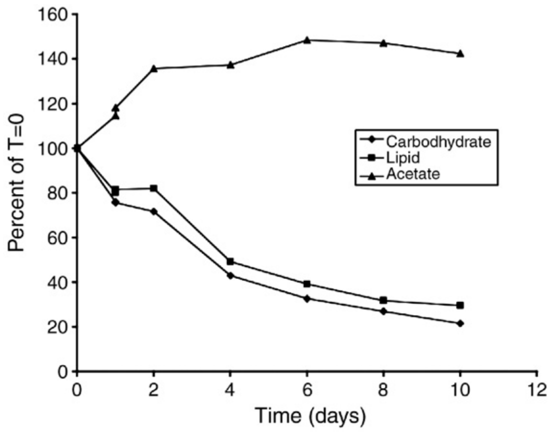

Fig. 5. Changes in the amount of carbohydrate (3–5.5 ppm), lipid (0.9–1.5 ppm) and acetate (2 ppm) over time with periodate oxidation of HSUR02. As oxidation takes place, the amount of carbohydrate and lipid decrease at approximately the same rate, suggesting much of the "lipid" fraction is 6-deoxy sugars. This is supported by the production of acetate, an oxidation product of 6-deoxy sugars, which increases during oxidation. The loss of carbohydrate and "lipid", and the increase in acetate were measured by changes in their respective in peak areas relative to benzoic acid.

samples, and 2.9% of the total organic carbon for the deep sea sample NPSG5200. The HSUR01 and HSUR02 surface samples also contained small amounts (0.5–2% total HMWDOC) of *N*-acetyl-glucosamine and *N*-acetyl-galactosamine.

Model carbohydrates, albumin, and pentanoic acid were used to experimentally verify the reaction stoichiometry for periodate over-oxidation from UV–Vis absorbance measurements (Table 2). Fig. 3A shows the time course of UV–Vis absorbance measurements for periodate oxidation of glucose, average glucopolysaccharide, albumin and pentanoic acid. Glucose was completely oxidized in <12 h, and yielded a reaction stoichiometry of 5:1 (moles of periodate: moles of glucose), in agreement with previous reports (Sklarz, 1967). Laminarin, amylose starch and maltooligoaccharide, all linear polysaccharides of glucose, were completely oxidized in 2–4 days, and yielded an average reaction stoichiometry of 6.2±0.5 with a range of 5.8 to 6.7 (Table 2). Bovine serum albumin reacted very slowly, with very little amino acid oxidation (~10%) taking place over a 10 day span. BSA had a reaction stoichiometry of 0.6:1 periodate to amino acid, significantly less than that of glucose or glucose polysaccharides. The value for albumin is well below the theoretical yield for complete oxidation, and shows that as expected, proteins are not extensively oxidized by periodate under our experimental conditions (Clamp and Hough, 1965; Pascual and Herráez, 1985; Pascual et al., 1989). The relatively low consumption of periodate by albumin, along with the relatively small amount of proteins in our HMWDOM samples (<8% HMWDOC; Aluwihare et al., 2005) indicates that only a small amount of periodate will be consumed by HMWDOM protein oxidation. Finally, we observed no reaction of periodate with pentanoic acid under our experimental conditions; rather, periodate absorbance at 230 nm increased slightly (4%) over the two weeks of reaction (Fig. 3A).

Results for the periodate over-oxidation of HMWDOM samples are given in Fig. 3B and Table 3. Periodate oxidizes all HMWDOM samples. Deep HMWDOM was oxidized at similar rates to surface HMWDOM, and the periodate consumed per mole of C (periodate: C ratio) is the same for all samples. For all three HMWDOM samples, the moles of periodate required for complete oxidation (1.3) is larger than the ratio determined for glucose (0.8) and glucose linear polysaccharides (1.0; Table 2).

Proton NMR spectra of the HSUR02 treated with periodate were collected to monitor the compositional changes within the HMWDOM during the course of over-oxidation (Fig. 4). Quantitative changes in the amount of carbohydrate (5.5–3.0 ppm), acetate (2.0 ppm), and "lipid" (1.7–0.9 ppm) were measured against benzoic acid (multiplets at 8.0 ppm, 7.6 ppm and 7.4 ppm), which was added as an internal standard immediately before spectral acquisition. Fig. 4A shows the HSUR02 sample before the addition of periodate, Fig. 4B after one hour of oxidation, Fig. 4C after 2 days of oxidation, and Fig. 4D after 10 days of oxidation. The amount of anomeric (O–C–O), alcohol (C–OH) and "lipid" (CH_{2-3}) hydrogens decreased during periodate oxidation, while formic acid (8.2 ppm), methanol (3.3 ppm), and acetic acid (2.0 ppm) appeared as oxidation products. Very little carbohydrate or "lipid" remained after 10 days of oxidation. A small decrease in formic acid was observed late in the experiment due to deuterium–hydrogen exchange with the D_2O solvent. Based on changes in ^{1}HNMR peak areas, periodate over-oxidation removes at least 70% of the carbohydrate and lipid peaks over 10 days of oxidation, while the amount of acetic acid in the sample increases by approximately 48% (Fig. 5). The increase in acetic acid during the course of the reaction is due to the production of new acetic acid as an oxidation product, most likely from 6-deoxy sugars.

4. Discussion

Periodate oxidation of glucose is a simple reaction that requires one mole of periodate to oxidize each carbon–

carbon bond (Whelan, 1964; Fig. 2A). The reaction of periodate with polysaccharides is more complicated, as illustrated for maltotriose in Fig. 2B. To fully oxidize a polysaccharide, periodate is consumed in the oxidation of glycosidic linkages, resulting in an apparent over-oxidation (e.g. more than one mole of periodate is consumed per carbon–carbon bond). In the over-oxidation reaction, the oxidation of polysaccharides proceeds as with glucose until all easily oxidized vicinal alcohols are oxidized, as shown in the second structure in Fig. 2B. Simple oxidation would stop at this point, with the resulting polyaldehyde chain as the product. To continue the reaction, oxidation of a carbon attached to an activated hydrogen occurs, introducing another hydroxyl group that allows the oxidation to continue. This results in the oxidative removal of one monomer unit from the glucose chain. Repetition of the last two steps in the reaction sequence gradually oxidizes the entire polysaccharide to formic acid, formaldehyde, and carbon dioxide.

In our hands, periodate over-oxidation of glucose and glucose linear polysaccharides proceeds as expected. The moles of periodate consumed per mole of carbon oxidized is 0.8 for glucose, between 1.0–1.1 for glucose linear polysaccharides, and 1.2–1.3 for HMWDOM. This higher periodate:carbon stoichiometric ratio for HMWDOM relative to linear polysaccharides may be indicative of a higher degree of branching and cross-linking within HMWDOM. If one additional mole of periodate is needed to oxidize each glycosidic bond, as suggested by the stoichiometry of periodate to glucose equivalents for glucose linear polysaccharides, then HMWDOM carbohydrate has on average 2–3 branch points per sugar. Linkage analyses of neutral sugars does indeed show that this fraction of HMWDOM is highly branched, consistent with our periodate oxidation results (Aluwihare et al., 1997).

Hedges et al. (2001, 2002) used solid-state ^{13}CNMR spectra of suspended and sinking particles to make quantitative estimates of the amount of carbohydrate, protein, and lipid in fresh and degraded particulate organic matter (POM). Organic matter compositional analyses based on NMR spectra rely on the assignment of particular signals within the ^{13}CNMR spectrum to functional groups characteristic of specific biochemical classes (Hedges et al., 2002; Nelson and Baldcock, 2005; Sannigrahi et al., 2005; Hertkorn et al., 2006). In some instances, the spectral interpretation is supported by quantitative molecular level analyses of simple sugars, amino acids, and lipids isolated after acid or base hydrolysis. However, even for fresh phytoplankton, molecular level analyses of sugars and lipids recover less carbon than derived from NMR based estimates. The discrepancy between molecular based and NMR based distributions increases for HMWDOM and for highly degraded particulate organic matter (POM), where only a minor fraction of the total organic carbon is recovered in molecular level analyses. NMR spectroscopy based compositional analyses of HMWDOM and highly degraded POM therefore rely on the untested assumption that the functional group assignments derived from fresh phytoplankton are appropriate for more degraded types of organic matter.

The aliphatic or "lipid" region of the ^{13}CNMR spectra not associated with acetamide represents 7% of the carbon for sample HSUR02 (Fig. 1). However, acid or base (saponification) hydrolysis of HMWDOM releases <1% of HMWDOC as fatty acids, sterols, alcohols and other lipids that can be measured by GC–MS (Mannino and Harvey, 1999), and we only recovered trace amounts of lipids from HSUR02. Even these low levels of lipid might only be *associated* with HMWDOM rather than *chemically bound* into the HMWDOM fraction (Boehm and Quinn, 1973). Aluwihare (1999) dissolved HMWDOM in low carbon deionized water, adjusted the pH to <3, and extracted the solution with organic solvents prior to saponification. Pre-extraction of HMWDOM in this way lowers the yield of lipid to trace levels. Aluwihare also collected ^{1}HNMR spectra of the organic solvent extract of HMWDOM after saponification. These spectra show only trace levels of lipid in the solvent extract, consistent with the GC–MS results. Aluwihare (1999) concluded that a large fraction of lipids recovered from HMWDOM are only loosely associated with this fraction of dissolved carbon, rather than bound through ester linkages. Acid and base hydrolysis will only release lipids that are bound in HMWDOM as esters (RO–C(O)–R′). Lipids that are bound as ethers (C–O–C) will remain in the HMWDOM (aqueous) fraction. To measure ether bound lipids, Aluwihare (1999) further treated HMWDOM with BBr_3, a well established technique for releasing ether bound lipids. No lipids were recovered using this approach, suggesting the lipid fraction of HMWDOM is not comprised of aliphatic ethers. Some algae synthesize insoluble cell wall material that has a significant lipid character. Pyrolysis MS and direct thermal ionization MS (DT–MS) analyses of HMWDOM does show the presence of alkyl phenols which are attributed to cell wall material (Boon et al., 1998). The very low amount of aromatic carbon observed in the ^{1}H- and ^{13}CNMR spectra of HMWDOM however suggests that alkyl phenols contribute only a small fraction of HMWDOM "lipid" carbon.

The “lipid” resonance in the ^{1}HNMR spectra of HMWDOM appears at 1.3 ppm, and in earlier papers we assigned this signal as a combination of methyl (CH_3) and methylene (CH_2) protons from deoxysugars and lipids respectively (Aluwihare et al., 1997, 2002; Repeta et al., 2002). Resonances for methine (6–7 ppm) and aromatic (7–8 ppm) protons (HC=C) are nearly absent from the ^{1}HNMR spectrum, suggesting that the lipid fraction is largely saturated. Periodate will slowly oxidize unsaturated and aromatic lipids, but will not oxidize saturated lipids (House, 1972). Irrespective of how lipids are bound into HMWDOM, periodate over-oxidation of HMWDOM should release lipids by removing the polymer to which they are associated. However, we were unable to detect lipids in the organic extracts of periodate oxidized HMWDOM. Further, periodate over-oxidation removes the lipid resonance from the ^{1}HNMR spectrum at nearly the same rate that the carbohydrate resonance is removed, and coincident with the increase in acetic acid (Fig. 5). Both observations suggest that the NMR spectroscopy-defined “lipid” fraction of HMWDOM is not attributable to saturated lipids, but instead arises from 6-deoxy sugars.

We attribute a large fraction of the resonance at 1.3 ppm in the ^{1}HNMR and at 17 ppm in the ^{13}CNMR spectra of HMWDOM to the methyl group on the 6-deoxy sugars. Integration of the peak at 17 ppm in the ^{13}CNMR spectrum of HSUR02 (Fig. 1) yields up to 3% of the total carbon as the 6-deoxy sugar methyl group, or up to 18% of the total carbon as 6-deoxy sugars. We further assign the resonance at 23 ppm as the methyl group in acetamide bound as *N*-acetyl amino sugars (Fig. 1). Integration of the 23 ppm peak yields 3.8% of the total carbon as the acetamide methyl C, in agreement with the yield of acetic acid from HMWDOM (6–8% C; Aluwihare et al., 1997; Aluwihare et al., 2005). Mao et al. (2003) report NMR spectra from extracts of anaerobically digested biosolids collected at a wastewater treatment plant that are similar to seawater HMWDOM, and interpret the aliphatic carbon at 24 and 19 ppm as arising from acetamide and peptidoglycan associated lactic acid respectively. Our data suggest the 19 ppm methyl signal may arise from deoxysugars. Further investigations comparing the chemical composition of wastewater HMWDOM and marine HMWDOM are warranted.

Our interpretation is supported by the net production of acetic acid during periodate oxidation coincident with the loss of 1.3 ppm signal. During periodate oxidation, the 5 carbon of 6-deoxy sugars is oxidized to a carboxylic acid, releasing acetic acid as an oxidation product. Periodate oxidation also releases acetic acid from *N*-acetyl hexosamines as the carbohydrate portion of the polysaccharide is destroyed. We observe a net increase of 40–50% in the amount of acetic acid during the oxidation process above what we would expect from acetamide hydrolysis alone. The 40–50% net increase of acetic acid results from the oxidation of 6-deoxy sugars. The amount of 6-deoxy sugars estimated by integration of the ^{13}CNMR spectrum at 17 ppm and the excess yield of acetic acid after periodate over-oxidation are in general agreement (3% vs 1.3%), but better estimates could be made by quantitative analyses of acetic acid using molecular methods (Albert and Martens, 1997).

^{1}HNMR spectra of sample NPSG5200 show the presence of carbohydrate and acetate, but a considerably larger portion of the spectrum appears as unresolved components that cannot be assigned to any particular class of major biochemicals. Yields of neutral sugars recovered from NPSG5200 after acid hydrolysis are only 2.9% HMWDOC, a smaller fraction of the total carbohydrate than found in surface water samples. ^{13}C NMR spectra of other deep sea samples likewise show relatively lower contributions from carbohydrate and larger contributions from carboxylate and “lipid” carbon. However, periodate oxidation of NPSG5200 proceeds rapidly and consumes the same amount of periodate on a per carbon basis as surface water HMWDOM. Major products include methanol and acetic acid. Periodate oxidation suggests that a large portion of highly degraded HMWDOM is carbohydrate, and that this carbohydrate is enriched in 6-deoxy and methyl sugars.

We also observed production of methanol with periodate oxidation of HMWDOM in both surface and deep water samples. Methanol is a product of periodate oxidation of methylated sugars (Hirano et al., 1974), and our results show that methylated sugars contribute a large fraction of the uncharacterized carbohydrate in HMWDOM. The ratio of methanol to acetic acid in the HSUR02 ^{1}H NMR spectra after oxidation is 1:3. While all of the methanol is produced by periodate over-oxidation, only a third of the acetic acid is generated from 6-deoxysugars (the remaining two thirds arises from acetamide hydrolysis). Therefore, the ratio of methanol produced from the oxidation of methyl sugars and acetic acid produced from the oxidation of 6-deoxy sugars is approximately 1:1, and methyl and 6-deoxy sugars are equally abundant in surface water HMWDOM. We did not perform quantitative analyses of NPSG5200 oxidation products to measure the yield of acetic acid and methanol, but such analyses could be useful in determining the contribution of 6-deoxy and methyl sugars to deep sea HMWDOM. Pyrolysis MS analyses of HMWDOM has previously shown that deoxy- and methyl sugars are present in HMWDOM (Minor et al.,

2001, 2002). Our results from periodate oxidation support these assessments, and further show that methyl and 6-deoxy sugars are quantitatively important in HMWDOM carbohydrate particularly in the fraction that is not depolymerized by treatment with acid.

Periodate oxidation provides additional evidence that the portion of HMWDOM carbohydrate not recovered using conventional hydrolytic techniques is compositionally different from the acid hydrolysable fraction. Acid hydrolysis releases approximately 10–20% of the total HMWDOM carbohydrate as largely seven neutral sugars (Table 1), including the 6-deoxy sugars rhamnose (11%) and fucose (19–20%), along with smaller amounts of hexosamines (5–6%) and uronic acids. The non-hydrolyzable portion of HMWDOM carbohydrate, however, is rich in *N*-acetyl amino sugars (25–30%; Aluwihare et al., 2005) and our analyses here show this fraction to be rich in methyl sugars as well. Preliminary estimates of methyl and 6-deoxy sugar content, based on the recovery of methanol and acetic acid after complete oxidation of HMWDOM, suggest that each type of sugar comprises at least 15% of the total carbohydrate. The relative contribution of 6-deoxy sugars to HMWDOM carbohydrate may therefore be somewhat less than the contribution of fucose and rhamnose to total neutral sugars (30–31%; Table 1) recovered after hydrolysis. Methyl and 6-deoxy sugars may occur together as methylated 6-deoxy sugars, or as distinct sugars. Further analyses, employing larger numbers of samples and higher precision techniques for measuring methanol and acetic acid would be useful to better quantify the contribution of methyl and 6-deoxy sugars to HMWDOM.

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References

Albert, D.B., Martens, C.S., 1997. Determination of low-molecular-weight organic acid concentrations in seawater and pore-water samples via HPLC. Mar. Chem. 56, 27–37.

Aluwihare, L.I., 1999. High molecular weight (HMW) dissolved organic matter (DOM) in seawater: chemical structure, sources and cycling. Ph.D. Thesis, Massachusetts Institute of Technology/ Woods Hole Oceanographic Institution, Woods Hole, MA.

Aluwihare, L.I., Repeta, D.J., Chen, R.F., 1997. A major biopolymeric component to dissolved organic carbon in surface sea water. Nature 387, 166–169.

Aluwihare, L.I., Repeta, D.J., Chen, R.F., 2002. Chemical composition and cycling of dissolved organic matter in the Mid-Atlantic Bight. Deep-Sea Res. II 49, 4421–4438.

Aluwihare, L.I., Repeta, D.J., Pantoja, S., Johnson, C.J., 2005. Two chemically distinct pools of organic nitrogen accumulate in the ocean. Science 308, 1007–1010.

Benner, R., Pakulski, J.D., McCarthy, M., Hedges, J.I., Hatcher, P.G., 1992. Bulk chemical characteristics of dissolved organic matter in the ocean. Science 255, 1561–1564.

Bock, K., Thøgersen, H., 1982. Nuclear magnetic resonance spectroscopy in the study of mono- and oligosaccharides. Annu. Rep. NMR Spectrosc. 13, 1–57.

Boehm, P.D., Quinn, J.G., 1973. Solubilization of hydrocarbons by the dissolved organic matter in seawater. Geochim. Cosmochim. Acta 37, 2459–2477.

Boon, J.J., Klap, V.A., Eglinton, T.I., 1998. Molecular characterization of microgram amounts of oceanic colloidal organic matter by direct temperature-resolved ammonia chemical ionization mass spectrometry. Org. Geochem. 29, 1051–1061.

Borch, N.H., Kirchmann, D.L., 1997. Concentration and composition of dissolved combined neutral sugars (polysaccharides) in seawater determined by HPLC-PAD. Mar. Chem. 57, 85–95.

Brown, F., Halsall, T.G., Hirst, E.L., Jones, J.K.N., 1948. The structure of starch. The ratio of non-terminal to terminal groups. J. Chem. Soc. 5, 27–32.

Clamp, J.R., Hough, L., 1965. The periodate oxidation of amino acids with reference to studies on glycoproteins. Biochem. J. 94, 17–24.

Dixon, J.S., Lipkin, D., 1954. Spectrophotometric determination of vicinal glycols. Anal. Chem. 26, 1092–1093.

Druffel, E.R.M., Williams, P.M., Bauer, J.E., Ertel, J.R., 1992. Cycling of dissolved and particulate matter in the open ocean. J. Geophys. Res. Oceans 97, 15639–15659.

Fukuda, R., Ogawa, H., Nagata, T., Koike, I., 1998. Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. Appl. Environ. Microb. 64, 3352–3358.

Hay, G.W., Lewis, B.A., Smith, F., 1973. Periodate oxidation of polysaccharides: General procedures. In: Whistler, R.L., BeMiller, J.N. (Eds.), Methods in Carbohydrate Chemistry, 5, pp. 357–361.

Hedges, J.I., Baldock, J.A., Gélinas, Y., Lee, C., Peterson, M., Wakeham, S.G., 2001. Evidence for non-selective preservation of organic matter in sinking marine particles. Nature 409, 801–804.

Hedges, J.I., Baldock, J.A., Gelinas, Y., Lee, C., Peterson, M.L., Wakeham, S.G., 2002. The biochemical and elemental compositions of marine plankton: A NMR perspective. Mar. Chem. 78, 47–63.

Hertkorn, N., Benner, R., Frommberger, M., Schmitt-Kopplin, P., Witt, M., Kaiser, K., Kettrup, A., Hedges, J.I., 2006. Characterization of a major refractory component of marine dissolved organic matter. Geochim. Cosmochim. Acta 70, 2990–3010.

Hirano, S., Fukuda, T., Sato, M., 1974. Periodate oxidation of some carbohydrates as examined by NMR spectroscopy. Agric. Biol. Chem. 38, 2539–2543.

House, H.O., 1972. Modern Synthetic Reactions. W.A. Benjamin, Inc., pp. 353–359.

Mannino, A., Harvey, H.R., 1999. Lipid composition in particulate and dissolved organic matter in the Delaware Estuary: sources and diagenetic patterns. Geochim. Cosmochim. Acta 63, 2219–2235.
McCarthy, M., Hedges, J., Benner, R., 1996. Major biochemical composition of dissolved high molecular weight organic matter in seawater. Mar. Chem. 55, 281–297.
Mao, J.-D., Hundal, L.S., Schmidt-Rohr, K., Thompson, M.L., 2003. Nuclear magnetic resonance and diffuse-reflectance infrared Fourier transform spectroscopy of biosolids-derived biocolloidal organic matter. Environ. Sci. Technol. 37, 1751–1757.
Minor, E.C., Boon, J.J., Harvey, H.R., Mannino, A., 2001. Estuarine organic matter compositon as probed by direct temperature-resolved mass spectrometry and traditional geochemical techniques. Geochim. Cosmochim. Acta 65, 2819–2834.
Minor, E.C., Simjouw, J.-P., Boon, J.J., Kerkhoff, A.E., van der Horst, J., 2002. Estuarine/marine UDOM as characterized by size-exclusion chromatography and organic mass spectrometry. Mar. Chem. 78, 75–102.
Nelson, P.N., Baldcock, J.A., 2005. Estimating the molecular composition of a diverse range of natural organic materials from solid-state ^{13}C NMR and elemental analyses. Biogeochemistry 72, 1–34.
Niemann, C., Hays, J.T., 1940. The structure of *N*-acetyl-D-glucosamine. J. Am. Chem. Soc. 62, 2960–2961.
Panagiotopoulos, C., Sempere, R., 2005. Analytical methods for the determination of sugars in marine samples: a historical perspective and future directions. Limnol. Oceanogr. Methods 3, 419–454.
Pascual, R., Herráez, M.A., 1985. The mechanism of the oxidative deamination and decarboxylation of serine and threonine by periodate in acid medium. Can. J. Chem. 63, 2349–2353.
Pascual, R., Herráez, M.A., Calle, E., 1989. Kinetics and mechanism of the oxidation of proline by periodate. Can. J. Chem. 67, 634–638.
Parrish, F.W., Whelan, W.J., 1959. Determination of molecular weights of polysaccharides by over-oxidation with periodate. Nature 183, 991–992.
Pazur, J.H., 1986. Neutral polysaccharides. In: Chaplin, M.F., Kennedy, J.F. (Eds.), Carbohydrate Analysis, A Practical Approach, pp. 55–96.
Perry, M.B., MacLean, L.L., 1999. Structural characterization of the antigenic O-chain of the lipopolysaccharide of *Escherichia coli* serotype O65. Carbohydr. Res. 322, 57–66.
Repeta, D.J., Quan, T.M., Aluwihare, L.I., Accardi, A., 2002. Chemical characterization of high molecular weight dissolved organic matter in fresh and marine waters. Geochim. Cosmochim. Acta 66, 955–962.
Sannigrahi, P., Ingall, E.D., Benner, R., 2005. Cycling of dissolved and particulate organic matter at station Aloha: insights from ^{13}C NMR spectroscopy coupled with elemental, isotopic and molecular analyses. Deep-Sea Res., Part I, Oceanogr. Res. Pap. 52, 1429–1444.
Sklarz, B., 1967. Organic chemistry of periodates. Quart. Rev. Chem. Soc. 21, 3–28.
Wakeham, S.G., Pease, T.K., Benner, R., 2003. Hydroxy fatty acids in marine dissolved organic matter as indicators of bacterial membrane material. Org. Geochem. 34, 857–868.
Whelan, W.J., 1964. Determination of reducing end-groups. In: Whistler, R.L. (Ed.), Methods in Carbohydrate Chemistry, 4, pp. 72–78.
Yamada, N., Tanoue, E., 2003. Detection and partial characterization of dissolved glycoprotiens in oceanic waters. Limnol. Oceanogr. 48, 1037–1048.